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Category	Document citation with notation, when necessary, of relevant parties	Claim concerned	
X	EP-A-0 381 601 (EASTMAN KODAK CO.) "In entirety"	1-8	
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Y	WO-A-8 400 691 (AMERICAN HOSPITAL SUPPLY CORP.) "Summary"	9	
A	EP-A-0 236 069 (CETUS CORP.) "In entirety"	1-10	
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The present invention concerns a diagnostic tool for application to bi logical fields such as human health, animal health or agriculture. In human and animal health, such a diagnostic tool is used f r detecting genetic diseases r infection by a pathogen. In the agricultural realm, it applies to the detection of contaminants such as pathogenic microorganisms in foodstuffs.

For example, a viral infection in an individual cannot generally be revealed by immunological tests. In fact, in most cases, it is the antibodies directed against the virus which are detected, which are indirect proof of the presence of the virus. In certain special cases in which the viremia is significant, the viral antigens may be directly detected in the blood of individuals. This is the case, for example, in the diagnosis of an infection by the hepatitis B virus (HBV). Generally, the immunological tests are only moderately successful due to their lack of sensitivity and specificity.

The most common immunological tests are capable of detecting 10^5 to 10^6 viral particles per milliliter. For the HBV, a serum is considered infectious with only 10^2 viral particles per milliliter (Prince et al., 1983). The number of false negative results is thus significant, as the threshold for infectiousness is at least 1000 times lower than the sensitivity threshold for the immunological tests.

The lack of specificity of these tests is directly related to the immunological reactions involved. False positive results are thus such a problem that a second immunological test, known as the "confirmation test", is often employed. This approach is particularly employed in the detection of the virus responsible for AIDS (Acquired Immune Deficiency Syndrome).

Research is currently under way concerning molecular biology methods allowing the diagnosis of an infection by a pathogen with a sensitivity and a specificity far superior to those obtained with the immunological tests. In addition, these methods based on molecular hybridization of nucleic acids provide direct proof of the presence of the pathogen, as it is its own genetic material which is detected.

Such a diagnostic tool, based on molecular hybridization, results in the detection of at least one defined sequence of deoxyribonucleic acid (DNA) or of ribonucleic acid

as a matrix. More specifically, an amplification cycle is compose of three stages. The first stage accomplishes the denaturing of the double-strand DNA sequences at 90 - 100 °C. The second stage results in the specific hybridization of oligonucleotides permitted by a decrease in the temperature during the phase of hybridization of the primer segments. The third stage accomplishes the extension of the hybridized oligonucleotide at a temperature close to the optimal temperature for the functioning of the DNA polymerase (37°C for the Klenow fragment and 72°C for a thermoresistant DNA polymerase extracted from Thermus *aquiticus*).

During the amplification cycle (n+1), the single-strand DNA sequences are synthesized by utilizing the sequences synthesized in the (n) cycle, as well as the sequences present at the end of the (n-1) cycle, as matrices. A sequence synthesized in the nth cycle thus cannot serve as a primer segment in the (n+1) cycle unless its length is at least equal to the distance in bases separating the two primer segments. Under these conditions, the number of DNA sequences specifically amplified varies exponentially with respect to the number of cycles.

The amplification of RNA sequences requires the preliminary stage of the synthesis of the complementary DNA (cDNA). This approach is important for the detection of the genome of retroviruses such as the human immunodeficiency virus (HIV) responsible for AIDS in humans (Ou et al., 1988).

The molecular hybridization reaction between two nucleic acid sequences is directly linked to the temperature. This hybridization, based on the complementarity of bases between two sequences, may occur between two DNA molecules, between two RNA molecules, or between a molecule of DNA and a molecule of RNA. The molecular hybridization thus allows either the pairing of two distinct molecules via hydrogen bonds, or the intra-molecular pairing between two complementary sequences. In the latter case, there is the formation of secondary structure in the DNA or RNA molecules. The influence of the temperature on the hybridization reaction is essential and each DNA (or RNA) sequence is characterized by its Tm, or the temperature at which 50% of the sequences are paired to the complementary sequences. The Tm of a precise sequence

may be determined experimentally by using spectrophotometry to monitor the hyperchromicity at 260 nanometers which accompanies the unpairing (r denaturation) of two complementary DNA sequences. All of the DNA sequences are in the single-strand form at high temperatures (100°C) and in the double-strand form at low temperatures (10-20°C).

The Tm of a DNA sequence depends ultimately on the two following parameters: the base sequence of the DNA in question and the ionic strength of the medium. Values for Tm generally vary from 20 to 85°C. Thus, all the molecular hybridization reactions must be performed under perfectly defined and controlled thermal conditions.

For biological diagnosis (human health, animal health, agriculture), a defined sequence of DNA or RNA may be detected by utilizing the complementary DNA or RNA sequence as a molecular probe. The probe is previously labeled, either by a radioactive isotope or some chemical modification. After hybridization between the target sequence and the probe sequence, the latter may be detected due to its labeling.

Using current technology, it is possible to detect the presence of a single molecule of DNA (deoxyribonucleic acid) or of RNA (ribonucleic acid) in a biological sample by using the PCR technique in conjunction with molecular hybridization with the aid of a nucleic probe. In other words, tests based on molecular hybridization are extremely sensitive, to the extent that a single pathogen may be detected in a sample (Wong et al., 1987). Compared to an immunological test, there is approximately a 10,000-fold gain in sensitivity.

The specificity of molecular hybridization reactions is excellent, as it is possible to detect a tiny variation in the base sequence composing the genetic material of an individual. The modification of a single base among the 3,500,000,000 bases which compose the human genome may thus be detected.

Despite the great specificity of the hybridization reactions, an ultra-sensitive diagnostic test associating the utilization of PCR and molecular hybridization with the aid of a nucleic probe does not have good specificity. In fact, it is not rare to obtain false positive results which significantly decrease the overall specificity of the test. This

phenomenon is due to accidental molecular contamination of the negative samples with at least one molecule of the target DNA or RNA. False positive results are ultimately a direct consequence of the great sensitivity of the test. In other words, the utilization of an amplification technique as powerful as PCR reveals molecular contaminants which previously went undetected.

The extreme sensitivity attained by diagnostic tests based on PCR is thus at the root of a new type of false positive result linked to molecular contaminants. The degree of the problem is such that it is now necessary to carry out different stages of the test at different sites in an attempt to minimize this phenomenon. This partitioning allows the physical separation of the pre-PCR manipulations, performed on samples which contain little specific DNA, from the post-PCR manipulation, performed on samples which are very rich in specific DNA. A minimum of three independent rooms (but preferably 4), with their own security airlocks, have begun to be installed by a large number of research laboratories worldwide. These rooms are either pressurized or de-pressurized, depending on whether the stage performed in them is pre-PCR or post-PCR, respectively.

This partitioning allows a significant reduction in the number of false positive results, but does not totally eliminate them. At any rate, this approach is costly, and makes a test which should be simple actually quite complex.

A certain number of devices are now commercially available which only execute one of the 3 or 4 stages which constitute a diagnostic test utilizing PCR and molecular hybridization with the aid of a nucleic probe. The only stages thus automated are the stage in which nucleic acids are extracted from the biological sample and the stage of amplification of the DNA sequences by PCR.

To accomplish the stage in which nucleic acids are extracted from the biological sample, only one device is currently available on the world market. It is a costly apparatus which can treat only a very small number of samples (a maximum of 8) in about 6 hours. Together, these characteristics make this device suitable for only very specialized research laboratories.

Between 12 and 15 different devices provide automated performance f the stage in which DNA sequences are amplified by PCR. All of these machines apply different temperatures to disposable and independent plastic tubes with capacities ranging from 0.75 ml to 1.50 ml. Each tube contains a biological sample to be treated. Two devices have also been proposed with an adapter capable of receiving a micro-titration plate.

Generally, existing devices have been designed to automatically perform a precise technological stage. None of them allow the performance of an entire diagnostic test by molecular hybridization. Thus the consecutive use of a device for extracting nucleic acids and a PCR device requires manipulations to be performed on each sample between the two automated stages. Such a manual intervention is necessary between these two stages and during the stages performed after the PCR.

These manipulations consist principally of opening and closing the disposable tubes with detachable stoppers, and of precision pipetting. During the manipulation of the tubes, the experimenter wears latex or vinyl gloves to ensure the greatest degree of cleanliness and especially to avoid contaminating the sample by nucleases present on his or her own skin. Moreover, when a tube is open, the significant danger of contamination is augmented by the presence of nearby tubes containing potential molecular contaminants. During the pipetting of a determined quantity of liquid from a donor tube and the delivery of this liquid to a receptacle tube, the pipetted liquid may be the sample itself, a buffer solution, a preparation containing at least one active principle (enzymes, oligonucleotides, triphosphate nucleotides, etc...).

For each biological sample treated, 12 to 15 pipettings must be carried out with the aid of a precision pipetting device for the single pre-PCR stage consisting of the extraction of nucleic acids. The precision pipetting device is very frequently the origin of the molecular contamination between two different reacting mixtures. Faced with this major problem, experimenters utilize positive-displacement pipetting systems more and more frequently; they seem to slightly decrease the frequency of contamination, but they are expensive.

In any case, all of these manipulations are a constant danger in terms of molecular contaminati n which could generate false positive test results. This situation is n

The main objective f the present invention is to completely eliminate the problems f molecular contamination during the course of automated treatment of the biological sample, which includes an *in vitro* stage of amplification f a specific DNA or RNA sequence. Such a result may be attained by using a procedure to amplify the number of at least one specific sequence of nucleic acids or of its complementary sequence, hereafter referred to as target sequences, in a biological sample, characterized by the fact that, on one hand, it includes the application of active principles to said sample and at least one preliminary stage, intended to increase the accessibility of nucleic acids present in the sample, followed by an amplification stage; on the other hand, said sample as well as the active principles are confined to the interior of a hermetically sealed container for the duration of the procedure. In order to address the molecular contamination problems in the most effective manner, the procedure may advantageously be characterized by the fact that the sampling is carried out directly in the aforementioned hermetically sealed container.

Preferably, such a procedure allows the performance of a complete biological diagnostic test, including, on one hand, a preliminary stage which increases the accessibility of the DNA or RNA to molecules contained in the initial sample and at least one active principle, such as DNA and RNA polymerization enzymes and DNA and RNA sequences which are partially or completely complementary; on the other hand, at least one additional stage subsequent to the amplification stage which is designed to detect the possible presence in the sample of all or part of at least one target sequence of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), the number of which has increased during the amplification stage.

The aforementioned amplification stage preferable employs, on one hand, the activity of a DNA polymerase or an RNA polymerase responsible for the synthesis of amplified DNA or RNA sequences; on the other hand, either at least one DNA or RNA sequence which is recognized as a primer by the aforementioned enzyme and is partially or completely complementary to a portion of the specific target sequence to be amplified, or a perfectly specific promoter sequence recognized by the enzyme and belonging to the target sequence to be amplified.

treatment and that said means consist of a rigid element having at least two spatial positions, such that the "closed" position allows the flexible tube to be compressed at a given location so as to impede the flow of fluid, and such that the other position, known as the "open" position, does not have this compression effect and allows the flow of fluid in this part of the tube.

Advantageously, several arrays may be grouped together to form a cartridge, allowing the simultaneous treatment of several samples with the usual means for ensuring thermal regulation, movement and selective flow of the samples and the active principles.

The device for the implementation of the procedure described in this invention consists, on one hand, of an array containing the active principles necessary for smooth functioning of the procedure; on the other hand, of means to ensure the movement, the thermal regulation and the selective flow of the sample and the active principles inside said array.

The present invention will be more easily understood with the aid of the complements to the description which follow, which refer to the attached diagrams in which:

- figure 1 represents a schematic view of a partial cross-section of an array which equips a preferred form of realization of the device according to the invention.

- figures 2, 3 and 4 represent cross-sectional views of certain components of the array represented in figure 1. They concern the base plate equipped with a network of grooves (figure 2), tubes formed by the association of the base plate and a perforated plate (figure 3), and the same tubes as those represented in figure 3 but cut at a point of connection of the perforated plate with a treatment container (figure 4).

- figure 5 is a schematic representation of the system of tubes forming the connections between the treatment containers of the array represented in figure 1, said system being equipped with valves.

- figures 6, 7, 8 and 9 represent different functional loops in the network shown in figure 5, each loop being associated with a configuration for opening and closing the valves.

- figure 10 represents a schematic view of a cartridge which equips a preferred form f realization of the device according to the invention.

The array, completely or partially represented in figures 1, 2, 3, and 4, as well as the cartridge represented in figure 10, are for single usage. This approach allows the use, inside said array and cartridge, of a hermetically sealed space containing active principles which have never been in contact with a potential source of contamination which could adversely affect the functioning of the procedure. The re-utilization of any portion of the array increases the risk of molecular contamination of the sample at any given time during the treatment, even if the part re-utilized is not on the path of the sample or of any liquid having been in contact with the sample. This is the case, for example, in a device composed of two interconnected parts, on one hand of a part for single-use, inside which all the manipulations of the sample and the active principles take place; on the other hand, of a reusable portion consisting of a cartridge containing a gas which, due to its pressure, accomplishes the movement of the sample and the active principles. Although the disposable portion may include, near its connectable end, a filtration system which impedes the passage of previously discussed molecular contaminants, the risks of contamination are greater than those associated with the utilization of a single-use array.

The aforementioned array (figures 1 to 4) and cartridge (figure 10) are designed to effectuate a complete diagnostic test, defined as a biological procedure allowing the realization of all the stages prior to amplification, the amplification itself and the stages subsequent to amplification, ending with a final qualitative, semi-quantitative or quantitative analysis. The aforementioned final analysis is intended to demonstrate the possible presence in the sample of at least one specific DNA or RNA sequence for which the number is increased during the amplification stage.

Advantageously, the introduction of the initial sample into the array or cartridge would occur upon sampling from an individual, an animal or an agricultural product. The aforementioned introduction could however be accomplished from a storage compartment serving as an intermediary between the sampling and the treatment effectuated on the sample in the array or cartridge.

The aforementioned array (figures 1 to 4) and cartridge (figure 10) are characterized by the fact that the hermetically sealed space is surrounded by a physically impermeable barrier, preferably made of plastic material, which may possess hermetic junction zones between two distinct constituent elements. These junction zones may result from gluing, thermofusing or an assemblage "by force" of the two aforementioned elements. Due to the construction materials utilized, the array does not allow the passage of molecular contaminants from the exterior to the interior, nor from the interior to the exterior. The passage of such molecules could disturb the treatment of the sample, particularly if it involves a DNA or RNA sequence of length greater than or equal to two nucleotides. It is particularly important to prevent the passage of DNA or RNA sequences the number of which would be susceptible to amplification during treatment of the sample.

The array (figure 1) consists of a cover (5) fixed to a base plate (1), which itself rests on a perforated flexible plate (2). At the end of the plate (1), two outlets each receive a flexible tube (E7, 4), each with an interior diameter of 1.2 mm. The cover (5) protects 6 elements, (E1), (E2), (E3), (E4), (E5) and (E6) attached to the plate (1). The elements (E7) and (4) are connected to a container (E8) which has a window (3).

The biological procedure which takes place inside this array includes at least one preliminary stage intended to increase the accessibility of nucleic acids contained in the initial samples. These nucleic acids will thus be more accessible to at least one molecule which is either present in the sample at the time of this stage and comes from the initial sample or from the addition of an active principle, or added as an active principle during a subsequent stage. The increase in the accessibility of nucleic acids may be obtained by either modification of molecules and biological structures contained in the sample, or by partial purification of the nucleic acids. This stage permitting an increase in the accessibility of nucleic acids is necessary since any living cell, be it a virus, a bacterium or a eukaryotic cell, contains biological structures more or less evolved to compartmentalize the cell so as to protect essential molecules such as nucleic acids. The accessibility of nucleic acids thus requires the partial or complete destruction of such structures, which are generally composed of proteins and lipids. The technique most

commonly empl yed consists of destroying or sufficiently modifying the molecules composing this biological structure. The destruction or the profound modification of structural proteins, either by a proteolytic enzyme such as proteinase K, or by the action of heat (treatment at 110°C) is a technique which is effective and perfectly compatible with the method and the device described in this invention.

The partial purification of nucleic acids may consist of the partial elimination of lipid and protein molecules contained in the sample. This method thus indirectly alters the aforementioned biological structures. The first technique consists of passing the sample through a column composed of a resin which has a high affinity for proteins of neutral pH and a very weak affinity for nucleic acids. A second technique consists of passing the sample at least once through an ion exchange column which will retain the nucleic acids due to ionic bonds between the negatively charged phosphate groups of the DNA or RNA and the cations fixed to the column. Later, a high-ionic strength elution buffer will be utilized to release the DNA or RNA by creating competition between the column anion and the salt anion (preferably Na^+) for the fixation of the negative charges of the phosphates. This simple technique is completely compatible with the method and device described in this invention. A third technique consists of extracting molecules such as lipids and proteins with the aid of organic solvents such as phenol and chloroform. A fourth technique consists of retaining the specific DNA or RNA sequences by hybridization with the at least partially complementary supported sequences present on a column.

The preliminary stage intended to increase the accessibility of nucleic acids present in the sample may be a simple heating of the latter to a temperature capable of denaturing certain proteins, namely a temperature which is preferably between 90°C and 110°C. In following such a protocol, the aforementioned preliminary stage may be merged with the first thermal treatment which denatures the DNA and RNA sequences during the amplification stage.

Advantageously, two successive preliminary stages are utilized during the process taking place in the array represented in figure 1. The first preliminary stage consists of

proteolytic treatment of the sample by proteinase K. T accomplish this, the sample is mixed with this enzyme in a container (E6), then it is moved to a container (E7) to be incubated at 70° C for 20 minutes. The second preliminary stage consists of passage of the sample, under low-ionic strength conditions, through an ion exchange column located in (E2). The nucleic acids retained on the column are then released with the aid of a high-ionic strength buffer located in (E1).

In the array represented in figure 1, it is advantageous, after the two successive preliminary stages intended to increase the accessibility of nucleic acids, to utilize the polymerase chain reaction with at least one oligonucleotidic primer carrying the fluorescent marker molecule at its 5' end. Thus, at least some of the sequences just synthesized during the amplification stage will carry said molecular marker and may be selectively recognized in a subsequent stage. All the active principles necessary for such an amplification stage are contained in (E5). After the addition of said active principles, the sample is moved to (E7), where it is subjected to different temperatures in a cyclical manner, allowing the PCR to be accomplished. From 15 to 40 PCR amplification cycles are generally sufficient to achieve a high degree of sensitivity for the diagnostic test. However, for the detection of certain pathogens like the HIV virus (type 1 and 2) and the hepatitis C virus (Garson et al., 1990), it is necessary to carry out a second PCR in using oligonucleotidic primers within the specifically amplified sequence. This approach allows a high degree of sensitivity to be achieved even when the initial number of pathogens is very small.

According to other versions of the procedure described in the present invention, the amplification stage may utilize the activity of a DNA polymerase or an RNA polymerase responsible for the synthesis of the amplified sequences, and in which two groups of enzymes may be distinguished. The first group is composed of enzymes which require the presence of a DNA or RNA primer sequence. In fact, such enzymes utilize as a substrate a duplex molecule made by hybridization between a target sequence, or matrix sequence, and a primer sequence carrying a free hydroxyl (OH) on the 3' end. The enzyme thus extends the primer sequence from its 3' end by incorporating, for a given

position on the target sequence, the complementary nucleotide by utilizing free triphosphate nucleotides present in the surroundings. This results in the synthesis f a sequence which is complementary to the target sequence. The second group consists of enzymes which require the presence of a perfectly specified promoter sequence which they recognize and from which they begin the synthesis of a sequence which is complementary to the sequence located immediately on the 3' side of said promoter sequence, in utilizing free triphosphate nucleotides present in the surroundings. The RNA polymerases of the T7 and SP6 bacteriophages are enzymes which behave in this latter manner (Stoffet et al., 1988).

Advantageously, a molecular marker other than fluorescence may be employed to modify the oligonucleotidic primer used during the amplification stage of the procedure described in the present invention. In fact, two principal groups of molecular markers exist. The first group is composed of molecules which, under certain physico-chemical conditions, may emit a signal of a particular nature which could be a radioactive emission, fluorescence, luminescence, bio-luminescence and phosphorescence. The second group is composed of molecules for which at least one of their parameters is easily measurable. It may be a soluble or precipitable product from the degradation of a substrate, particularly by an enzyme such as alkaline phosphatase or peroxidase. The presence of a soluble degradation product may be detected by colorimetry. Due to the nature of their emission, degradation products exist for substrates belonging to the first group of molecular markers.

According to still other versions of the procedure described in the present invention, at least one oligonucleotidic primer utilized during the amplification stage may be modified in ways other than those resulting in the attachment of a molecular marker. In fact, a nucleic acid sequence may be modified by the addition of a radioactive isotope (P^{32}, S^{35}, H^{3}), of a chemical group, of a haptene, of a molecule of molecular weight greater than 300 daltons or of an enzyme.

The procedure which takes place in the array represented in figure 1 preferably includes a final stage for analysis of the specifically amplified sequences. To accomplish

this, the sample having been amplified is placed in contact with a supported DNA probe which is at least partially complementary to the amplified target sequence, then the presence of said amplified target sequence, specifically retained on said support, is evaluated with the aid of a device capable of quantitatively detecting the fluorescence emitted from a marker carried by the amplified sequences. The probe sequence is preferably an oligonucleotide consisting of 20 to 35 nucleotides covalently bound to a solid support made of silica.

The final stage of the aforementioned analysis consists first of at least one passage, at a precise temperature, of the sample through a column included in (E8) and containing a solid support on which at least one aforementioned probe sequence is held, then of the removal and return to solution, at an elution temperature, of at least one amplified target sequence. Due to the genetic variability of certain target sequences and thus to the approximate knowledge of the target sequence really contained in the samples, several elution temperatures may be advantageously utilized in the direction of increasing temperatures. At least one eluate may thus be submitted to the detection of the molecular marker carried by the amplified target sequence with the aid of a detector external to the array.

According to a simplified version of the procedure described in the present invention, the amplification stage represents the end of said procedure. Still other variations of the procedure described in the present invention may be utilized depending on the nature of the additional stage. Said additional stage may occur before or after the amplification stage, and may utilize at least one DNA or RNA probe sequence, modified or non-modified, which under certain physico-chemical conditions hybridizes specifically by complementarity between the bases with at least one target sequence. The aforementioned probe sequence may be present in the sample in free or supported form. The result of such an additional stage is to have at the end of the treatment a qualitative, semi-quantitative or quantitative analysis of the presence of at least one target sequence in the initial sample and thus to make available a complete diagnostic test which is automated and takes place under the previously defined conditions of isolation.

During said additional stage, the probe sequence may advantageously be added before the amplification stage and serves as a specific inhibitor during amplification. In fact, a DNA polymerase or an RNA polymerase is halted or strongly slowed if during the course of its relative progression on the matrix sequence, and as it synthesizes the complementary sequence, it runs into the 5' end of a DNA or RNA sequence, known as a bioquant sequence, hybridized to said matrix sequence. A probe sequence, complementary to at least one target sequence to be amplified, added before the amplification stage, acts as a bioquant sequence and thus has an inhibiting effect on the specific amplification of at least one target sequence. For a given sample, if the amplification stage is independently applied, on one hand, to said sample containing a probe sequence or bioquant sequence; on the other hand, to said sample not containing the probe sequence, the comparison of the analyses of the amplified sequences in these two cases verifies with certainty the presence or absence of the target sequence in the initial sample. To be effective, such a probe sequence must be modified at its 3' end, preferably by incorporation of a di-deoxynucleotide, so as not to be used as a primer by the DNA or RNA polymerase during amplification.

During said additional stage, the probe sequence may preferably be added after the amplification stage and specifically hybridized to at least one amplified target sequence. In this approach, a very large number of different hybridization models may be utilized, provided that the probe sequence is immobilized on a solid or free support, depending on the number of different probe sequences utilized and depending on the type of possible modification to at least one target sequence or at least one probe sequence. The hybridization model utilized determines the following stages of the treatment of the sample for the final stage is an analysis of the amplified sequences.

Advantageously, when the aforementioned hybridization model and the final analysis have as their goal to distinguish at least one amplified target sequence among the collection of other sequences present in the sample, it is necessary to utilize either means capable of distinguishing these molecules by at least one of their physico-chemical parameters, or a hybridization complex between said amplified target sequence and at least one complementary probe sequence. The physico-chemical parameter most often

utilized is the molecular weight of a DNA or RNA sequence, or its length as a number of nucleotides, but its evaluation requires the use of a separative technique such as electrophoresis or gel filtration.

Preferably, the procedure described in this invention allows a complete diagnostic test to be performed, the last stage being a qualitative, semi-quantitative or quantitative analysis of the presence of a given molecular marker, yielding a measure of the presence of at least one specifically amplified target sequence, given that a correlation exists, depending on the nature of the analysis, between the presence of the amplified target sequence and the presence of all or part of this target sequence in the initial sample. The aforementioned molecular marker may be carried by either all or part of an amplified target sequence, or by all or part of a probe sequence. The molecular marker is characterized by the fact that its presence may be directly measured with the aid of a detection apparatus.

For smooth implementation of the procedure taking place in the array shown in figure 1, certain containers (figure 1) would be advantageously provided with external means for heating and cooling. Thus, (E7) is equipped with three independent heating elements which, on one hand, allow the sample to be maintained at a constant temperature between 37°C and 70°C during the course of proteolytic treatment by proteinase K; on the other hand, allow the sample to be successively subjected to three temperatures associated with a cycle of amplification by PCR, accomplishing the denaturing of the double-strand DNA, the hybridization of the primers and the extension by a DNA polymerase. (E8) is also equipped with a heating element which allows the detachment of an amplified target sequence during the previously described analysis stage. Most of the stages composing the overall treatment of the sample require a dynamic system. This consists of movement of the sample between different containers and of movement of an active principle necessary for the treatment toward the sample. This is accomplished by the action of a peristaltic pump acting on the flexible wall of (E7).

According to still other versions of the device for the implementation of the procedure described in the present invention, the speed of movement f the sample or of the active principle may be continuous or discontinuous. The driving force necessary for such movement may come from different sources: fluid under pressure, displacement of a magnetic system, passive capillary effect, thermal gradient, peristaltic pump acting on the wall of a flexible tube, difference in pressure between the fluid (gas for example) in front of the sample and the fluid behind the sample (this difference in pressure being created by a mechanical system acting locally), or a combination of the preceding different possibilities.

Certain tubes (figure 5) of the array in question (figure 1) are equipped with means designed to permit or impede the flow of fluid from one container to another. These means are designed to permit or impede the flow of the sample, of at least one gas or at least one active principle at a given time in at least one specific tube so as to ensure the smooth progression of the treatment. To accomplish this (figures 2 through 4), the plate (2) may advantageously be flexible and have grooves (7) on one of its faces (figure 2). The pattern of the grooves was designed to satisfy precise functional needs. The close contact between plates (1) and (2), accomplished by thermofusion or gluing (figure 3), delimits the tubes exiting the grooves (7). The flexible structure of the plate (2) permits the play of cams (9) external to the array, which intermittently compress the protuberances (6) of the grooves (7). This acts to impede the flow of fluid in a given tube. The play of three cams (9) connected to a single support (8), shown on figure 3, allows three tubes to be simultaneously compressed, and thus impedes the circulation of fluids in the compressed region. Together, plates (1) and (2) form a well-defined system of tubes, to which the containers (E1), (E2), (E3), (E4), (E5), (E6) and (E7) and the flexible tube (4) are connected. The connections (figure 4) to the contalner elements (E) are ensured by a perforation (10) in the plate (1), which interlocks with a sleeve (11) containing a tube (12). The connection to the flexible tube (4) is ensured by a completely analogous system. A valve (X) (figure 5), corresponding to an external cam of the aforementioned type, may be in either the open position (X = 1) to permit the free flow of

fluid in the region, or the closed position (X = 0) to impede the circulation of fluid in the region.

The initial biological sample, in liquid form, is introduced to the container (E4), where is mixed with the active principles which make the viral DNA accessible. After mixing, the concentrations are as follows: 25 mM sodium acetate, pH 6.5; 2.5 mM EDTA; 0.5% SDS; 2.5 mg/ml proteinase K. The system of valves (figure 5) being in the configuration [X1 = 0, X2 = 0, X3 = 1, X4 = 0, X5 = 0, X6 = 0] (figure 6), the sample moves towards (E7) in traversing the connections (a16) and (a18). Inside the container (E7), the sample is brought to 70_ C during a 15 minute interval. The system of valves being in the configuration [X1 = 0, X2 = 0, X3 = 1, X4 = 0, X5 = 0, X6 = 0], the sample returns to (E6) in passing through (a18) and (a16). The system of valves being in the configuration [X1 = 0, X2 = 0, X3 = 1, X4 = 0, X5 = 0, X6 = 0], the sample flows from (E6) toward (E2) in passing through (a15) and (a5). In (E2), the sample flows through an ion exchange column which specifically retains nucleic acids. The eluate not retained on the column flows from (E2) to (E4) in passing through (a3) and (a9). Due to the spatial configuration of the connections (a9) and (a10), the eluate is blocked in (E4). The system of valves being in the configuration [X1 = 1, X2 = 1, X3 = 0, X4 = 0, X5 = 1, X6 = 0] (figure 7), the elution buffer contained in (E1) is brought to (E2), where it flows through the ion exchange column and releases the nucleic acids. The liquid sample thus formed by this elution is diluted, then it flows from (E2) toward (E5) in passing through (a4) and (a12). Due to the spatial configuration of the connections (a11) and (a12), the sample is blocked in (E5), where it is mixed which the active principles required for the PCR amplification. After mixing, the concentrations are as follows: 200 µM deoxyadenosine-5'triphosphate; 200 µM deoxythymidine-5'triphosphate; 200 µM deoxyguanosine-5'triphosphate; 200 µM deoxycytidine-5'triphosphate; 250 nM of each oligonucleotidic primer; 10mM Tris-HCl, pH 8.4; 1.5 mM magnesium chloride; 100µg/ml gelatin and 1 unit per 50 µl thermoresistant DNA polymerase (Taq polymerase, for example). The system of valves being in the configuration [X1 = 0, X2 = 0, X3 = 0, X4 = 0, X5 = 1, X6 = 1] (figure 6), the sample flows from (E5) toward (E7), passing through (a14) and (a18), where it undergoes the thermal treatment necessary for the PCR

amplification stage. Means for heating and cooling external to the array allow the sample contained in (E7) to be subjected to 30 successive thermal cycles defined as follows: 1 cycle = 1 minute at 35°C, then 1 minute at 50°C, then 1 minute at 72°C. The system of valves being in the configuration [X1 = 0, X2 = 0, X3 = 0, X4 = 0, X5 = 1, X6 = 1], the sample flows from (E7) toward (E8) in passing through (a19) and (a21). Inside the container (E8), equipped with external means for heating, the sample is placed in contact with an oligonucleotidic probe attached to a solid support and the specific amplified sequences are selectively retained while (E8) is maintained at 60°C. The eluate containing the non-specific sequences continues its migration toward (E4), passing through (a20), (a17) and (a10), where it is blocked due to the spatial configuration of the connections (a10) and (a11). The system of valves being in the configuration [X1 = 0, X2 = 0, X3 = 0, X4 = 1, X5 = 0, X6 = 1] (figure 9), the buffer contained in (E3) is brought to (E8), where it is placed in contact with the support retaining the specific DNA sequences to be detected. The container (E8) is thus subjected to a temperature accomplishing the dehybridization between the supported probe sequence and the amplified sequence to be detected. The latter is thus returned to solution and its presence directly detected inside (E8) with the help of a detector centered on the axis of the window (3). In a valve configuration identical to that previously mentioned and after its analysis by the detector, the sample continues its migration toward (E4), where it is blocked due to the configuration of (a10) and (a8). (E4) is a trap container designed to accumulate the products extracted from the sample and the previously used active principles. (E4) has 4 connections, (a8), (a9), (a10) and (a11), allowing the free flow of gas between any two of these connections. However, its compartmentalized structure allows the collection of the amplified and analyzed sample within an element which is not contaminated by other liquids resulting from the treatment. The amplified sample may thus be later used by simple sampling from (E4).

Several arrays may be grouped together to form a cartridge (figure 10) which consists of 4 distinct parts: (C1), (C2), (C3) and (C4). The (C1) portion includes the containers (E1), (E2), (E3), (E4), (E5) and (E6). The (C2) portion is a flexible joint

structure between (C1) and (C3). The (C3) portion includes the container (E7) and the portion (C4) including the container (E8).

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CLAIMS

1) Procedure for numerical amplification of at least one specific sequence of nucleic acid or of its complementary sequence, hereafter referred to as target sequences, in a biological sample, characterized by the fact, on one hand, that it includes the action of active principles on said sample and at least one preliminary stage, intended to increase the accessibility of nucleic acids present in the sample, followed by an amplification stage; on the other hand, that said sample as well as the active principles are confined to the interior of a hermetically sealed container for the duration of the procedure.

2) Procedure according to claim 1, characterized by the fact that the sampling takes place directly in said hermetically sealed container.

3) Procedure according to claims 1 and 2, characterized by the fact that it is capable of performing a complete biological diagnostic test including, on one hand, a preliminary stage which increases the accessibility of DNA and RNA to molecules contained in the initial sample and at least one active principle, such as DNA and RNA polymerization enzymes and DNA and RNA sequences which are partially or totally complementary; on the other hand, at least one additional stage subsequent to the amplification stage which is designed to demonstrate the possible presence in the sample of all or part of at least one deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) target sequence, the number of which is increased during the amplification stage.

4) Procedure according to claims 1 through 3, characterized by the fact that the amplification stage employs, on one hand, the activity of a DNA polymerase or of an RNA polymerase responsible for the synthesis of the amplified DNA or RNA sequences; on the other hand, either at least one DNA or RNA sequence recognized as a primer by the aforementioned enzymes which is partially or totally complementary to a portion of the specific target sequence to be analyzed, or a perfectly defined promoter sequence recognized by the enzyme and belonging to the target sequence to be amplified.

5) Procedure according to claims 1 through 4, characterized by the fact that at least one additional stage, occurring before or after the amplification stage, utilizes at least one DNA or RNA probe sequence, modified or non-modified, which under certain physico-chemical conditions specifically hybridizes by complementarity between the bases with at least one target sequence, and which is present in the sample in free or supported form.

6) Procedure according to claims 1 through 5, characterized by the fact, on one hand, that the amplification stage utilizes the polymerase chain reaction technique (PCR) to specifically increase the number of molecules of a specific target sequence with a modified oligonucleotidic primer which carries a molecule capable of emitting a measurable signal; on the other hand, that after amplification, the sample is placed in contact with a supported DNA probe which is at least partially complementary to the amplified target sequence and that the presence of said amplified target sequence specifically retained on said support is evaluated with the aid of a device external to the array which allows the quantitative detection of the signal-emitting molecule carried by the amplified sequences.

7) Device for the implementation of the procedure according to one of claims 1 through 6, characterized by the fact that it includes, on one hand, at least one container for the active principles necessary for the preliminary stage intended to increase the accessibility of nucleic acids; on the other hand, at least one container for the active principles necessary for the amplification stage, and that the containers are hermetically sealed to one another so as to constitute a unit, hereafter referred to as the array, delineating an enclosed space with means provided for the mixing and thermal regulation of the sample and the active principles.

8) Procedure according to claim 7, characterized by the fact that the ensemble defined by the hermetically sealed container and the active principles which it contains is for single usage, meaning it is disposable after the treatment of a single biological sample.

9) Device according to one of claims 7 and 8, characterized by the fact, on one hand, that the means partially or totally ensuring the movement of the sample and the active principles in the array consist of a peristaltic pump acting on the flexible wall of a tube which is part of the array; on the other hand, that the connections between the different containers are tubes equipped with means designed to permit or impede the flow of fluid at a given time through at least one specific tube so as to ensure the smooth progression of the treatment and that said means consist of a rigid element having at least two spatial positions, such that the "closed" position allows the flexible tube to be compressed at a given location so as to impede the flow of fluid, and such that the other position, known as the "open" position, does not have this compression effect and allows the flow of fluid in this part of the tube.

10) Device according to one of claims 7 through 9, characterized by the fact that several arrays are grouped together to form a cartridge, allowing the simultaneous treatment of several samples with the usual means for ensuring thermal regulation, movement and selective flow of the samples and the active principles.

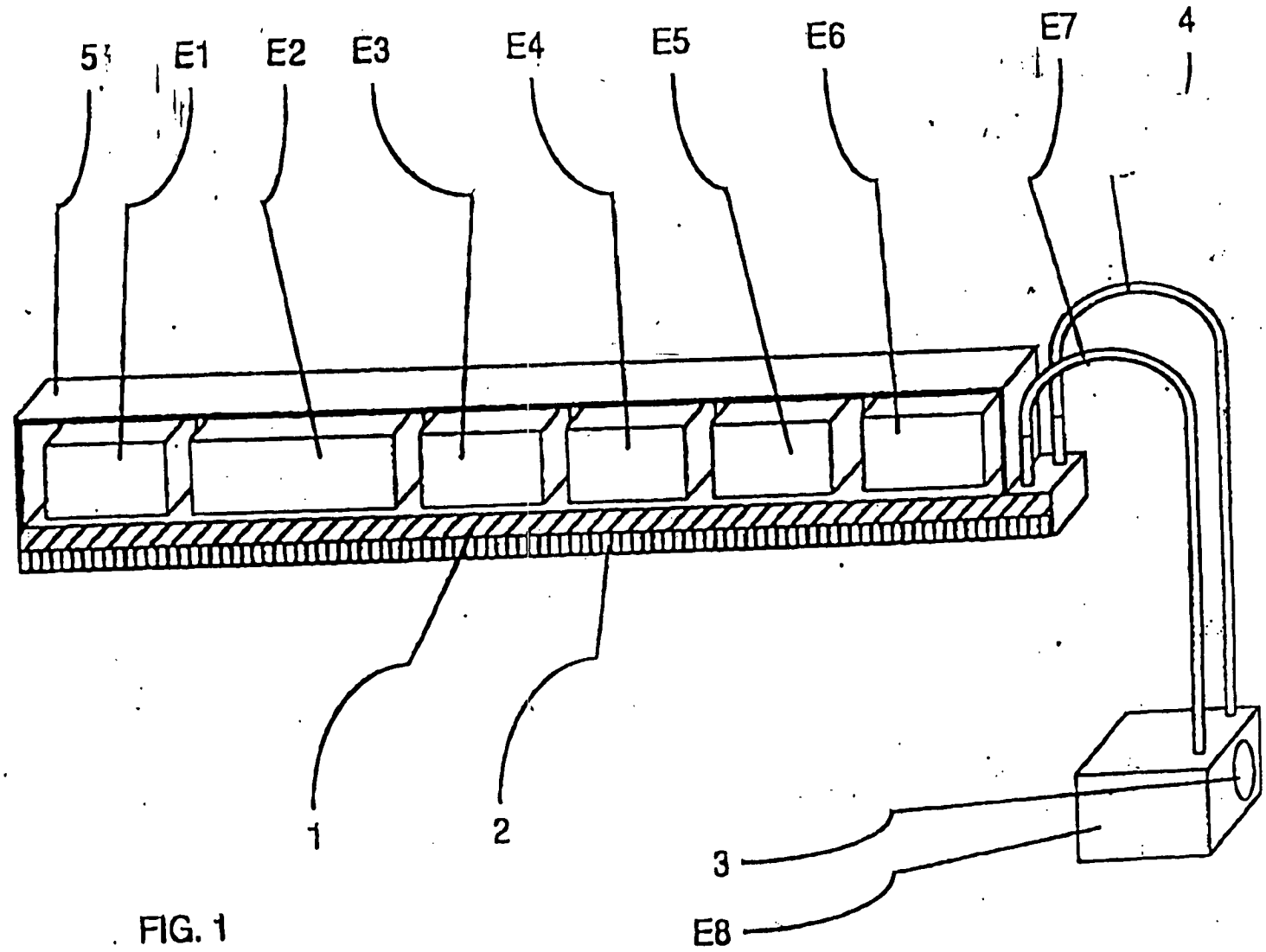

FIG. 1

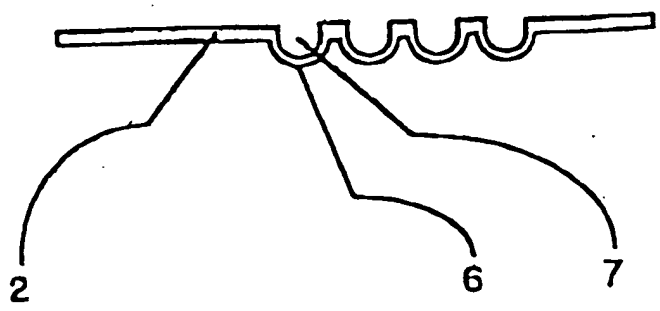

FIG. 2

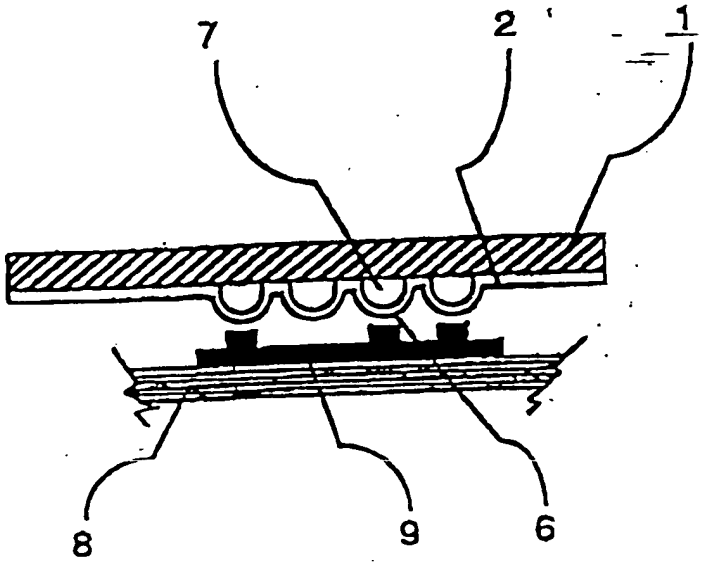

FIG. 3

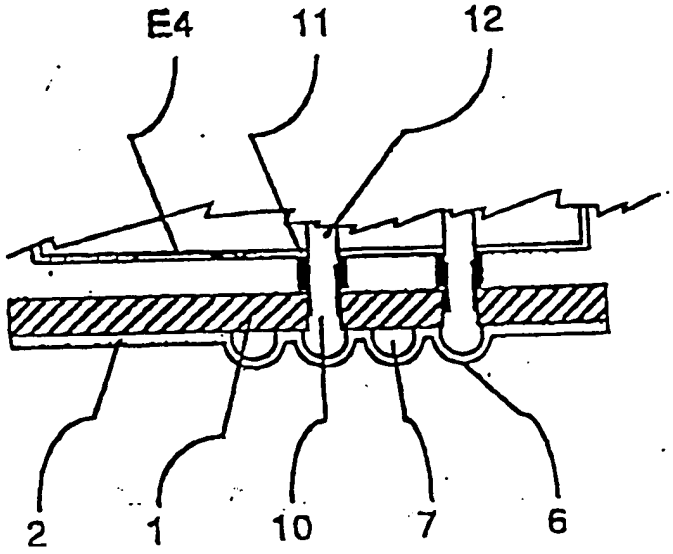

FIG. 4

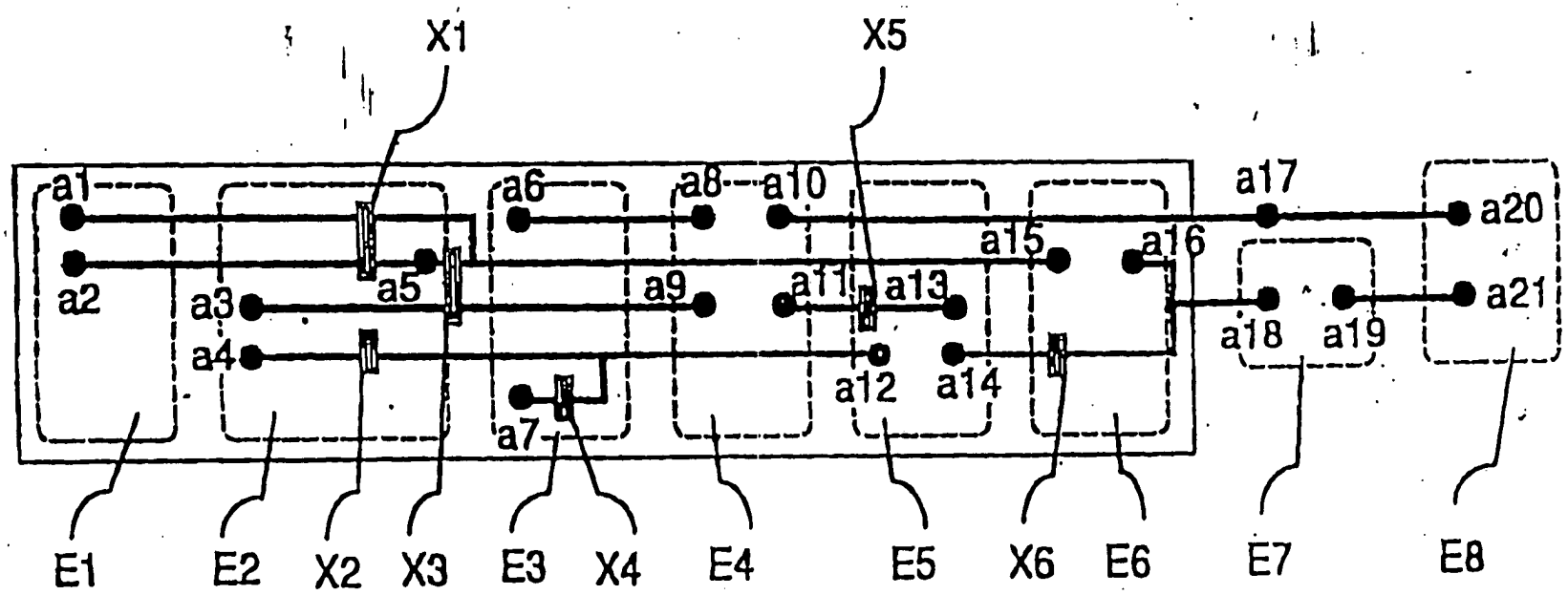
X1
X5
a1
a2
a3
a4
a5
a6
a7
a8
a9
a10
a11
a12
a13
a14
a15
a16
a17
a18
a19
a20
a21
E1
E2
X2
X3
E3
X4
E4
E5
X6
E6
E7
E8

FIG.5

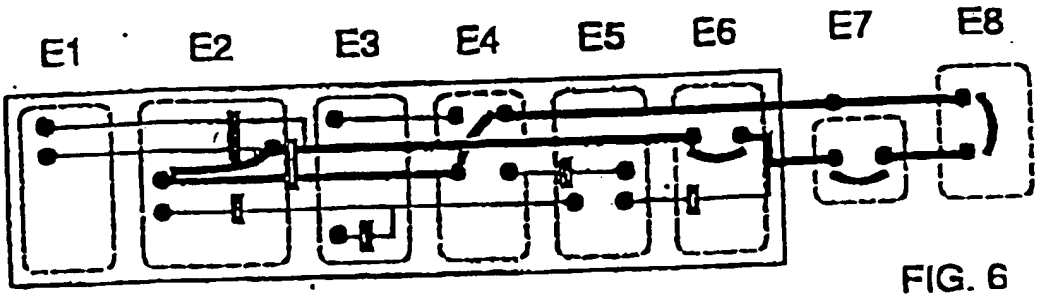

FIG. 6

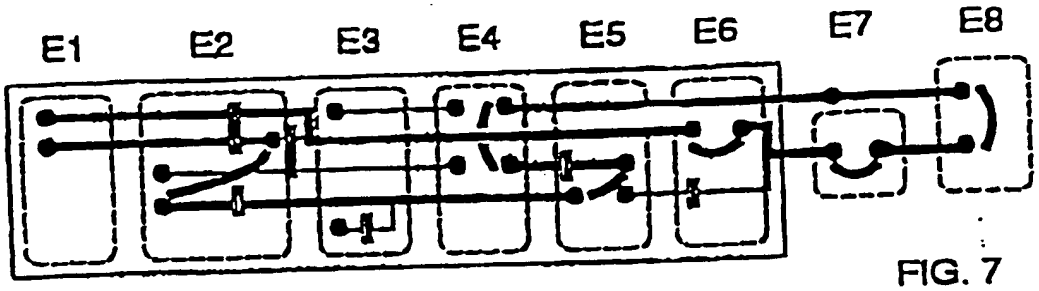

FIG. 7

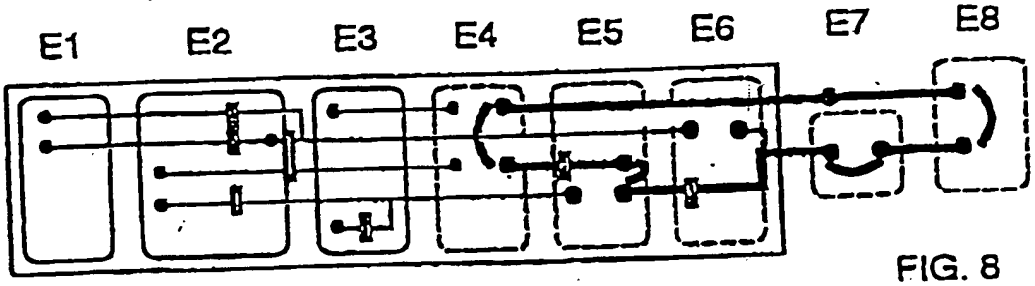

FIG. 8

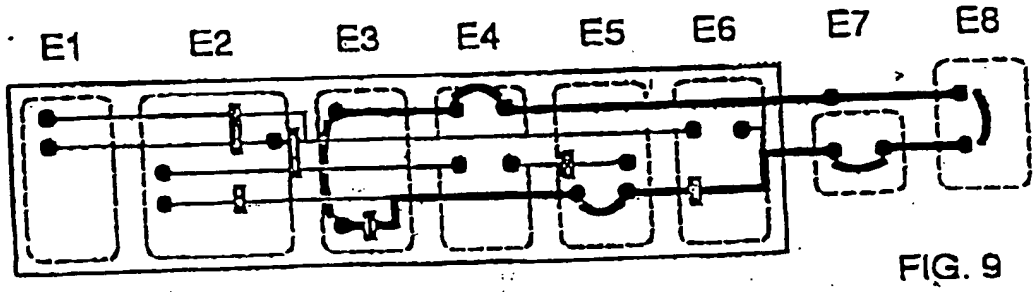

FIG. 9

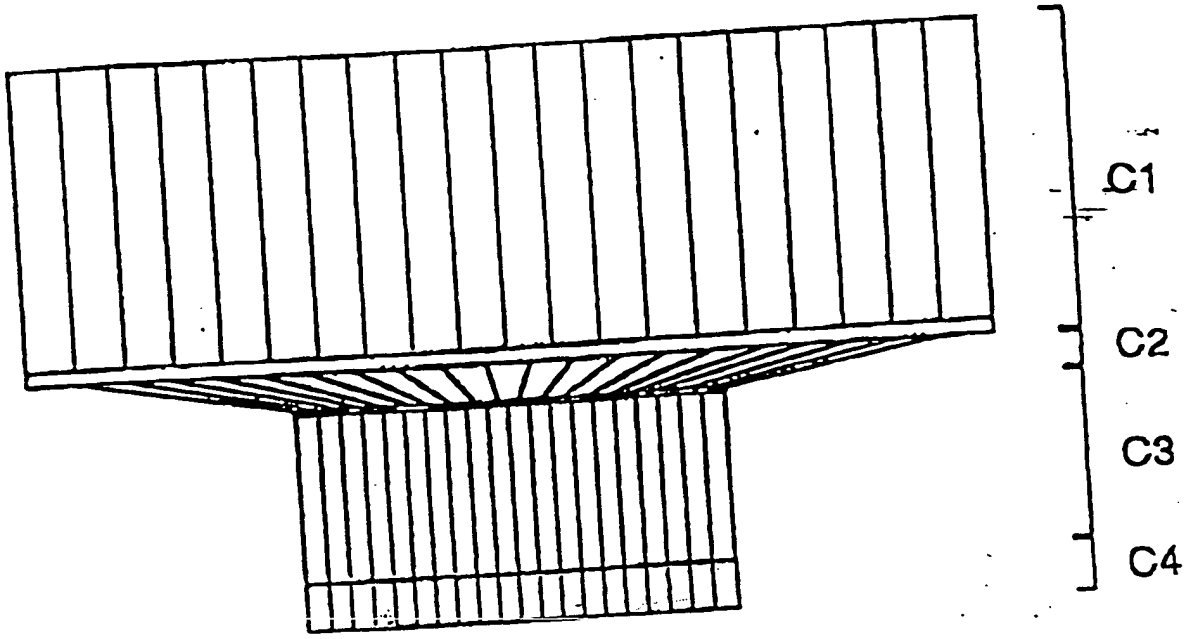

FIG.10